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(54) Title: REDUCED IRREVERSIBLE BOMBESIN ANTAGONISTS

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CH2-CH-CH2-CO-;        (I)
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(57) Abstract

A peptide of the formula (I): R-A-B-C-Trp-Ala-Val-X-Y-T-W, wherein R represents a group of the formula 4-$(ClCH_2CH_2)_2N$-C_6H_4-$CH_2CH(NHR_1)CO$-; 3-$(ClCH_2CH_2)_2N$-C_6H_4-$CH_2CH(NHR_1)CO$-; 4-$(ClCH_2CH_2)_2N$-C_6H_4-CO-; 3-$(ClCH_2CH_2)_2N$-C_6H_4-CO-; $ClCH_2CH_2NHCO$-; ClCH=CH-CO-, BrCH=CH-CO-, CH_2=CClCO-, CH_2=CBrCO- (either *cis* or *trans* isomers); (a); CH≡C-CO-; $ClCH_2CH_2CH_2N(NO)CO$-; $ClCH_2CO$-$CH(R_2)NHCO(CH_2)_2CO$-; A represents a valence bond, or a Gly, Leu-Gly, Arg-Leu-Gly, or Gln-Arg-Leu-Gly residue, B represents a valence bond or a Asn, Phe or Thr residue; C represents a Gln or His residue, X represents a Gly or ala residue; Y represents a valence bond, or a His(R_3), his(R_3), Phe, phe, Ser, ser, Ala or ala residue; T represents a valence bond, or a Leu, leu, Phe or phe residue; W represents a group of the formula OR_2, NH_2, $NH(CH_2)_4CH_3$, $NH(CH_2)_2C_6H_5$, Met-R_4, Leu-R_4, Ile-R_4 or Nle-R_4; R_1 represents a hydrogen atom, a Boc group or an acyl group, R_2 represents a hydrogen atom, a linear or branched alyphatic chain having from 1 to 11 carbon atoms, a benzyl or a C_1-C_{21} phenyl group, R_3 represents a hydrogen atom or a Tos, Dnp or Bzl group, R_4 represents NH_2, NH-NH_2 or OR_2, one or more peptide bonds (CONH) are replaced by reduced peptide bonds (CH_2, NH), and the pharmaceutically acceptable salts thereof and pharmaceutically acceptable salts are bombesin antagonists. Their preparation and pharmaceutical compositions containing them are also described.

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REDUCED IRREVERSIBLE BOMBESIN ANTAGONISTS

The present invention relates to peptide derivatives, to pharmaceutical compositions containing them, to processes for their preparation, and to their application as therapeutic agents.

In this specification symbols and abbreviations are those commonly used in peptide chemistry (see Eur.J. Biochem. (1984) 138, 9-37). Consequently, the three-letter amino acid symbols denote the L configuration of chiral amino acids. D-amino acids are represented by small letters: e.g., ala = D-Ala. Other symbols and abbreviations used are: AA, amino acid; Ac, acetyl; AcOEt, ethylacetate; BBS, bombesin; Boc, t-butoxycarbonyl; BuOH, n-butyl alcohol; BOP, benzotriazolyloxy-tris[dimethylamino]phosphonium hexafluorophosphate; Cab, [p-bis(2-chloroethyl)amino]benzoyl; dec., decomposition; DCC, N,N'-dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DCU, N,N'-dicyclohexylurea; DMAP, 4-dimethylaminopyridine; DMF, freshly distilled dimethylformamide; DMSO, dimethylsulfoxide; Dnp, 2,4-dinitrophenyl; EGF, epidermal growth factor; EtOH, ethyl alcohol; FAB (or FD)-MS, fast atom bombardment (or field desorption) mass spectrometry; ECC, ethylchlorocarbonate; EI-MS, electron impact mass spectrometry; Et_2O, diethyl ether; Glp, L-pyroglutamic acid; h-GRP (or p-GRP), human (or porcine) gastrin releasing peptide; HCl/AcOH, anhydrous HCl in glacial acetic acid; HOBt, 1-hydroxybenzotriazole; I.D., internal diameter; HOSu, N-hydroxysuccinimide;

Mel, [bis(2-chloroethyl)amino]- L-phenylalanine; MeOH, methyl alcohol; m.p., melting point; mod., modification; n.d., not determined; Nle, L-norleucine; NMM, N-methylmorpholine; NMR, nuclear magnetic resonance; OSu, N-hydroxysuccinimidyl; Pd/C, palladium on charcoal; PE, petroleum ether 40°-70°; RP-HPLC, reversed phase high performance liquid chromatography; SCLC, small cell lung carcinoma; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin layer chromatography; Tos, p-toluensulphonyl; TsOH, p-toluensulphonic acid.

The capital letter psi -Ψ- between two amino acids indicates an amide bond replacement by the function specified between the brackets.

The invention provides a peptide of the formula I:

R—A—B—C—Trp—Ala—Val—X—Y—T—W I

wherein

R represents a group of the formula

4-$(ClCH_2CH_2)_2N-C_6H_4-CH_2CH(NHR_1)CO-$(pMel);

3-$(ClCH_2CH_2)_2N-C_6H_4-CH_2CH(NHR_1)CO-$(mMel);

4-$(ClCH_2CH_2)_2N-C_6H_4-CO-$(Cab); 3-$(ClCH_2CH_2)_2N-C_6H_4-CO-$;

$ClCH_2CH_2NHCO-$; $ClCH=CH-CO-$, $BrCH=CH-CO-$, $CH_2=CClCO-$,

$CH_2=CBrCO-$ (either <u>cis</u> or <u>trans</u> isomers);

$\underset{\diagdown\;O\;\diagup}{CH_2-CH}-CH_2-CO-$; $CH \equiv C-CO-$; $ClCH_2CH_2CH_2N(NO)CO-$;

$ClCH_2CO-CH(R_2)NHCO(CH_2)_2CO-$;

A represents a valence bond, or a Gly, Leu-Gly, Arg-Leu-Gly, or Gln-Arg-Leu-Gly residue,

B represents a valence bond or a Asn, Thr or phe residue;

C represents a Gln or His residue,

X represents a Gly or ala residue;

Y represents a valence bond, or a His(R_3), his(R_3), Phe, phe, Ser, ser, Ala or ala residue;

T represents a valence bond, or a Leu, leu, Phe or phe residue;

W represents a group of the formula OR_2, NH_2, $NH(CH_2)_4CH_3$, $NH(CH_2)_2C_6H_5$, Met-R_4, Leu-R_4, Ile-R_4, or Nle-R_4;

R_1 represents a hydrogen atom, a Boc group or an acyl group having from 1 to 11 carbon atoms.

R_2 represents a hydrogen atom, a linear or branched alyphatic chain having from 1 to 11 carbon atoms, a benzyl or a phenyl group. Preferred alyphatic chains which R_2 may represent include methyl, ethyl, n-propyl-, iso-propyl, n-butyl and iso-butyl groups.

R_3 represents a hydrogen atom or a Tos, Dnp or Bzl group, and

R_4 represents NH_2, NH-NH_2 or OR_2.

In addition, one or more peptide bonds (CONH) are replaced by reduced peptide bonds (CH_2NH).

Preferred acyl group which R_1 may represent are aliphatic acyl group such as acetyl, formyl, propionyl and butirryl or aromatic such as benzoyl optionally substituted by nitro, methoxy, amino group or halogen atoms.

Preferably in the formula I R represents pMel or Cab, R_1 represents hydrogen atom, Boc or acetyl group, A represents a valence bond, B represents a valence bond or phe residue, C represents a Gln residue, X represents a His(Dnp), His or Gly residue most preferably Gly, Y represents a valence bond, T represents a Leu residue, W represents a group of the formula Leu-NH_2 or Nle-NH_2 and the reduced peptide bond (CH_2NH) is that between T and W.

Salts of these peptides with pharmaceutically acceptable acids are within the scope of the invention. Such acid addition salts can be derived from a variety of inorganic and organic acids such as sulfuric, phosphoric, hydrochloric, hydrobromic, hydroiodic, nitric, sulfamic, citric, lactic, pyruvic, oxalic, maleic, succinic, tartaric, cinnamic, acetic, trifluoracetic, benzoic, salicylic, gluconic, ascorbic and related acids.

The synthesis of the peptides of the invention may be accomplished by classical solution methods. The synthesis consists essentially of appropriate successive condensations of protected amino acids or peptides. The condensations are carried out so that the resulting peptides have the desired sequence of amino acid residues.
The amino acids and peptides, which can be condensed according to methods known in peptide chemistry, have the amino and carboxyl groups, not involved in peptide bond formation, blocked by suitable protecting groups capable of being removed by acid or alkali treatment or by hydrogenolysis.
For the protection of the amino group the following protective groups may, for example, be employed: benzyloxycarbonyl, t-butoxycarbonyl, trityl, formyl, trifluoracetyl, o-nitrophenylsulphenyl, 4-methyloxybenzyloxycarbonyl, 9-fluorenylmethoxycarbonyl, 3,5-dimethoxy-α-α'-dimethylbenzyloxycarbonyl or methylsulphonylethoxycarbonyl.
For the protection of the carboxyl group the following protective groups may, for example, be employed: methyl, ethyl, t-butyl, benzyl, p-nitrobenzyl or fluorenylmethyl, amide, hydrazide, t-butoxycarbonyl hydrazide or benzyloxycarbonyl hydrazide.

The hydroxy functions of hydroxy amino acids and the imino function of histidine may be protected by suitable protecting groups (throughout all the synthesis or only during a few steps) or may be unprotected. For the protection of the hydroxy function the following protective groups may, for example, be employed; t-butyl, benzyl, acetyl. For the protection of the imidazole imino function the following groups may, for example, be used: 2,4-dinitrophenyl, tosyl, benzyl. De-protecting reactions are carried out according to methods known per se in peptide chemistry.

The condensation between an amino group of one molecule and a carboxyl group of another molecule to form the peptidic linkage may be carried out through an activated acyl-derivative such as a mixed anhydride, an azide or an activated ester, or by direct condensation between a free amino group and a free carboxyl group, in the presence of a condensing agent such as dicyclohexylcarbodiimide, alone or together with a racemization preventing agent, such as N-hydroxysuccinimide or 1-hydroxybenzotriazole, or together with an activating agent such as 4-dimethylamino-pyridine. The condensation may be carried out in a solvent such as dimethylformamide, dimethylacetamide, pyridine, acetonitrile, tetrahydrofuran or N-methyl-2-pyrrolidone.

The formation of a reduced peptide bond is accomplished by condensation of an N-protected amino acid aldehyde with a C-protected amino acid or peptide in the presence of a reducing agent, such as $NaBH_3CN$. The aldehyde, in turn, is usually obtained by condensing an N-protected amino acid with N,O-dimethylhydroxylamine, and reducing the resulting amide with a suitable reducing agent, such as $LiAlH_4$.

The reaction temperature may be from -30°C to room temperature. The reaction time is generally from 1 to 120 hours.

The scheme of synthesis, the protecting groups and condensing agents are selected so as to avoid the risk of racemization.

Biological activity

The peptides of the present invention are endowed with potent antagonism versus "in vitro" and "in vivo" effects induced by bombesin, such as contraction of smooth musculature, modification of behaviour of central origin and mitogenesis.

Bombesin (BBS) is a tetradecapeptide of formula Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2, originally isolated from the skin of a frog. The biological activity resides in the C-terminal part of the molecule: BBS(6-14)nonapeptide is as active as the parent compound. The human counterpart of bombesin is a 27 amino acid peptide, known as gastrin-releasing peptide (h-GRP). Bombesin and bombesin-like peptides display a number of biological activities (J.H. Walsh (1983) in "Brain Peptides", D.T. Krieger, M.J. Brownstein and J.B. Martin (eds), Wiley Interscience Publ., pp. 941-960), including autocrine growth-promoting effects on human small cell lung carcinoma (SCLC) (F. Cuttitta et al. (1985) Cancer Survey, 4, 707-727), autocrine and/or paracrine stimulation of human prostatic cancer cell proliferation (M. Bologna et al., Cancer, in press) and modulation of the EGF receptor (I. Zachary and E. Rozengurt (1985) Cancer Surveys, 4, 729-765).

A bombesin antagonist, by competing with the natural ligand for the receptor(s), would inhibit the triggering of the cascade of events leading to abnormal cell proliferation.

Different approaches in this direction have been followed by different research groups. A series of C-terminal bombesin nona- and decapeptides, characterized by amino acid deletion, inversion or substitution, has been the object of a previous patent application by our side (EP Patent Application n° 89102283.2). These peptides, however, like other BBS antagonists, usually show moderate affinity for the BBS receptor.

The compounds of the present invention, due to the alkylating moiety, display greater receptor affinity than the parent peptides, and behave as receptor antagonists either when given in combination with bombesin or when administered 24 hours before bombesin challenge. In addition, owing to the presence of reduced peptide bonds, water solubility and, in many cases, also antagonistic properties are increased.

Biological test results

The binding affinity of the compounds of the present invention for the bombesin receptors was determined on mouse Swiss 3T3 fibroblasts (I. Zachary and E. Rozengurt (1985) Proc. Natl. Acad. Sci. USA, 82, 7616-7620) (Table 1).

The effect on mitogenesis was determined in quiescent and confluent Swiss 3T3 cells maintained in serum free medium (A.N.Corps et al

(1985) Biochem J. 231, 781-785). In a first set of experiments, analogues were given alone or in combination with bombesin. In a second set of experiments, cells were pre-treated with the alkylating peptides, washed, left at 37°C for 24 hours and then challenged with bombesin. In both cases, DNA synthesis was evaluated as [H^3]thymidine incorporation (Table 2).

In addition, exposure to these peptides in the 0.1-50 μM range was associated with significant reduction in the growth of SCLC cell lines (such as NCI-H345, NCI-N592, NCI-H69, NCI-H128), as well as of prostatic carcinoma cell lines (such as DU145 and PC3) (Table 3).

Parenteral administration of these peptides at doses ranging between 1 ng/kg - 100 mg/kg to nude mice was associated with significant growth reduction of the above mentioned transplanted human SCLC and prostatic carcinoma cell lines.

TABLE 1

BINDING AFFINITY OF BOMBESIN

ALKYLATING ANALOGUES ON MOUSE SWISS 3T3 FIBROBLASTS

COMPOUND	IC_{50} (nM)*
I	839 ± 178
II	28 ± 1
III	2340 ± 291
IV	2.3 ± 1.0
V	0.9 ± 0.5

Reference peptides:

BBS	12.6 ± 0.65
Spantide	11100
[pro^2]Spantide	14000
[Leu$^{13}\psi$(CH_2-NH)Leu^{14}]BBS	214 ± 30

* mean value ± S.E.M.

TABLE 2

[H^3]THYMIDINE INCORPORATION IN MOUSE SWISS 3T3 FIBROBLASTS

COMPOUND	FOLD INCREASE OVER BASAL VALUE				% INHIBITION IN THE PRESENCE OF 25nM BBS			
					A		B	
	5nM	50nM	0.5μM	5 μM	0.5μM	5 μM	0.5μM	5 μM
I	n.d.	n.d.	1.8	1.7	54 ± 5	75 ± 4	69 ± 2	84 ± 3
II	n.d.	n.d.	0.8	0.8	86 ± 3	90 ± 3	55 ± 5	86 ± 6
III	n.d.	n.d.	0.8	0.7	34 ± 21	86 ± 1	46 ± 14	61 ± 16
IV	n.d.	n.d.	1.1	1.3	79 ± 7	85 ± 8	0	85 ± 7
V	n.d.	n.d.	1.1	0.9	83 ± 8	85 ± 7	0	39 ± 7
Reference peptides:								
BBS	3.0±1							
[$Leu^{13}\Psi(CH_2\text{-}NH)Leu^{14}$]BBS			1	1	29±10	56±4	0	0

A= analogues are given in combination with BBS

B= cells are pre-treated with analogues, washed, left at 37°C for 24 h and then challenged with BBS

TABLE 3

"IN VITRO" ACTIVITY OF ALKYLATING ANALOGUE ON SCLC CELL LINES

COMPOUND	IC_{50} (nM)	
	NCI-N592	NCI-H69
II	110	940
IV	700	783
Reference peptide :		
[Leu^{13} ψ (CH_2-NH)Leu^{14}]BBS	520	1,660

The peptides of the formula I, therefore, find application in the therapy of human neoplasms which are modulated in their growth and progression by peptides of the GRP family, either directly or in concert with other growth factors.

In addition, these alkylating analogues can be used in the management of the clinical symptoms associates with these deseases and due to hypersecretion of GRP-like peptides.

The compounds of the invention can be administered by the usual routes, for example, parenterally, e.g. by intravenous injection or infusion, or by intramuscular, subcutaneous, intracavity and intranasal administration.

The dosage depends on the age, weight and condition of the patient and on the administration route.

On the basis of the "in vitro" and "in vivo" data in mice it can be estimated that the therapeutic doses in humans will be in the range of 1 ng/kg - 100 mg/kg, once to 6 times daily.

Moreover, the toxicity of the peptides of the present invention is quite negligible.

The invention also provides pharmaceutical compositions containing a compound of formula (I) as the active substance, in association with one or more pharmaceutically acceptable excipients.

The pharmaceutical compositions of the invention are usually prepared following conventional methods and are administered in a pharmaceutically suitable form.

For instance, solutions for intravenous injection or infusion may contain as carrier, for example, sterile water or, preferably, they may be in the form of sterile aqueous isotonic saline solutions.

Suspensions or solutions for intramuscular injections may contain, together with the active compound, a pharmaceutically acceptable carrier, e.g., sterile water, olive oil, ethyl oleate, glycols (e.g., propylene glycol) and, if desired, a suitable amount of lidocaine hydrochloride.

Furthermore, according to the invention, there is provided a method of treating neuroendocrine neoplasms (such as small cell lung carcinoma and prostatic carcinoma) or the clinical symptoms associated with these diseases in patients in need of it, comprising administering to the said patients a composition of the invention.

Chemistry

Methods:

a) TLC was performed on pre-coated plates of silica gel 60 F_{254} (Merck), layer thickness 0.25 mm, length 20 cm, with the following eluents:

System A: n-butanol/acetic acid/water = 600/150/150 by volume

System B: chloroform/methanol= 99/1 by volume

System C: chloroform/methanol = 90/10 by volume

System D: toluene/ethyl acetate/acetic acid/water = 100/10/20/10 by volume.

b) Analytical RP-HPLC was performed on a Hewlett Packard Mod. 1084 apparatus on a LiChrosorb Hibar RP-18 column (Merck) 250 x 4 mm I.D., particle diameter 5 µ. The following eluents were used:

A= KH_2PO_4 20 mM, pH 3.5/acetonitrile 9/1 by volume;

B= KH_2PO_4 20 mM, pH 3.5/acetonitrile 3/7 by volume.

The elution was programmed with a linear gradient from 60% to 90% B over a period of 20 min (System A) or from 30 to 70% B over a period of 15 min (System B), and then isocratically for 15 min, with a flow rate of 1 ml/min.

The peptides were characterized by their retention time (RT).

c) Preparative RP-HPLC was performed using a Delta Prep 3000 apparatus (Waters) on a Deltapak column (Waters), 300 x 19 mm I.D., particle diameter, 10 µ. The following eluents were used:

A= 0.05% TFA in water;

B= 0.05% TFA in acetonitrile/water 7/3 by volume.

Flow rate= 24 ml/min; detection wavelength= 220 nm.

Elution methods are reported in the single examples.

In each case, fractions were checked by analytical RP-HPLC and those showing a purity greater than 98% were pooled. After removal of acetonitrile, the solutions were lyophilized.

d) Amino acid analysis was carried out on acid hydrolysates (either at 110°C for 22 h in 6 N HCl + 0.1% phenol or at 100°C

for 16 h in 3 N mercaptoethansulfonic acid, both under N_2). Only natural amino acid residues were determined. Due to partial decomposition in normal hydrolysis conditions, Trp was determined only in hydrolysates with mercaptoethansulfonic acid.

Example 1

Preparation of

Boc-pMel-Gln-Trp-Ala-Val-Gly-His(Dnp)-LeuΨ(CH_2NH)Met-NH_2 (I).

Step 1

Boc-Val-Gly-OBzl (Ia)

43.45 g (200 mmol) of Boc-Val-OH were dissolved in 500 ml of anhydrous THF, cooled at -25°C and treated with 22.48 ml (200 mmol) of NMM, followed by 19.80 ml (200 mmol) of ECC. After stirring for 2 min at -12°C, a pre-cooled solution of 67.47 g (200 mmol) of H-Gly-OBzl . TsOH and 22.48 ml (200 ml) of NMM in 500 ml of anhydrous DMF was added. The reaction mixture was stirred for 2 hours at - 12°C, then filtered from salts and the solution evaporated under reduced pressure. The oily residue was dissolved in 1200 ml of AcOEt and the solution washed successively with 10% citric acid (5 x 100 ml), brine, 5% $NaHCO_3$ (5 x 100 ml) and brine to neutrality. After drying over Na_2SO_4, the solvent was evaporated and the residue purified by flash-chromatography on silica gel, eluting with AcOEt/MeOH 95/5. 56.68 g (78% yield) of compound Ia were obtained from PE: m.p. 76-78°C; $[\alpha]^{25}_{D}$ - 28.0° (C 1, MeOH); FD-MS: m/z 365 (100, MH^+); Rf_B 0.70; RT_A 11.8.

Step 2

H-Val-Gly-OBzl . HCl (I b)

56.40 g (154.75 mmol) of Boc-Val-Gly-OBzl (Ia) were made to react for 30 min at room temperature with 570 ml of 1.33 N HCl/AcOH. The solvent was removed under reduced pressure and the oily residue evaporated twice from DMF and washed with Et_2O. 44.2 g (95% yield) of compound Ib were obtained as an oil: FD-MS: m/z 265 (100, MH^+) as free base; Rf_A 0.54; RT_B 6.7.

Step 3

Boc-Ala-Val-Gly-OBzl (Ic)

Starting from 27.81 g (147 mmol) of Boc-Ala-OH and 44.2 g (147 mmol) of H-Val-Gly-OBzl . HCl (Ib), and operating as described for the preparation of Ia, but replacing AcOEt with CH_2Cl_2 in the washings, 54.82 g (68% yield) of compound Ic were obtained from CH_2Cl_2/PE: m.p. 142-146°C; FD-MS: m/z 436 (100, MH^+); Rf_B 0.26; RT_A 9.4.

Step 4

H-Ala-Val-Gly-OBzl . HCl (Id)

Starting from 27 g (62 mmol) of Boc-Ala-Val-Gly-OBzl (Ic), and operating as described for the preparation of Ib, 22.65 g (98% yield) of compound Id were obtained from MeOH/AcOEt/PE: m.p. 178-181°C; FD-MS: m/z 336 (100, MH^+) as free base; Rf_A 0.53; RT_B 7.0.

Step 5

Boc-Trp-Ala-Val-Gly-OBzl (Ie)

The condensation was carried out as described for Ia, starting from 18.41 g (60.5 mmol) of Boc-Trp-OH and 22.50 g (60.5 mmol) of H-Ala-Val-Gly-OBzl (Id). The crude product was then dissolved in DMF and precipitated by dropping the solution with stirring at 0°C into a 10% citric acid acqueous solution. The precipitate was filtered and washed with water to neutrality, then dried at 40% over P_2O_5. 35.70 g (95% yield) of compound Ie were obtained: m.p. 154-177°C (dec.); FD-MS: m/z 621 (100, $M^{+\cdot}$); Rf_B 0.10; RT_A 13.2.

Step 6

H-Trp-Ala-Val-Gly-OBzl . HCl (If)

33.75 g (54.28 mmol) of Boc-Trp-Ala-Val-Gly-OBzl (Ie) were made to react for 30 min at room temperature with 340 ml of 1.33 N HCl/ AcOH, 34 ml of anisole and 17 ml of 2-mercaptoethanol. The solvents were removed under reduced pressure and the oily residue evaporated twice from DMF. The product was precipitated from MeOH/PE and washed several times with PE and then with Et_2O. 26.75 g (88% yield) of compound If were obtained: m.p. 118-122°C; FD-MS: m/z 521 (100, $M^{+\cdot}$) as free base; Rf_A 0.66; RT_A 5.8.

Step 7

Boc-Gln-Trp-Ala-Val-Gly-OBzl (Ig)

Starting from 11.73 g (47.66 mmol) of Boc-Gln-OH and 26.6 g (47.66 mmol) of H-Trp-Ala-Val-Gly-OBzl . HCl (If), and operating as described for Ie, 29.86 g (83% yield) of compound VII were obtained from MeOH/CH_2Cl_2/Et_2O/PE: m.p. 208-211°C (dec.); FD-MS: m/z 749 (100, $M^{+\cdot}$); Rf_C 0.51; RT_A 7.6.

Step 8

Boc-Gln-Trp-Ala-Val-Gly-OH (Ih)

53 ml of a solution composed by 12 ml (318 mmol) of 99% formic acid and 33 ml (300 mmol) of NMM in 1 l of MeOH were added with stirring to a suspension of 3 g (4 mmol) of Boc-Gln-Trp-Ala-Val-Gly-OBzl (Ig) and 1.86 g of 10% Pd/C in 80 ml of DMF.

The reaction mixture was stirred for 1 h at room temperature, the catalyst was filtered off and the solvent evaporated in vacuo. The residue was ground with AcOEt, giving 2.2 g (84% yield) of compound Ih: FD-MS: m/z 682 (100, MNa^{+}), 659 (40, $M^{+\cdot}$); Rf_A 0.52; RT_B 8.0.

Step 9

<u>Boc-Leu-N(CH_3)OCH_3 (Ii)</u>

24.93 g (100 mmol) of Boc-Leu-OH . H_2O were dehydrated by evaporation from 200 ml of DMF, and dissolved in 350 ml of CH_2Cl_2. 9.95 g (102 mmol) of HCl . HN(CH_3)OCH_3 and 3.05 g (2 mmol) of DMAP were added with stirring at 40°C, followed by a few drops of DMF to obtain an almost clear solution. A solution of 20.65 g (100 mmol) of DCC in 130 ml of CH_2Cl_2 and a solution of 11.24 ml (100 mmol) of NMM in 130 ml of CH_2Cl_2 were then dropped separately in 30 min, keeping the reaction temperature at 0°C. After an additional hour at room temperature, the reaction mixture was filtered from salts and DCU, and evaporated. The residue was dissolved in AcOEt, filtered from other DCU, and washed successively with 10% citric acid (5 x 100 ml), 5% $NaHCO_3$ (15 x 100 ml) and brine to neutrality. After evaporation of the solvent, the oily residue was purified by flash-chromatography on silica gel, eluting first with PE/Et_2O 85/15 (to remove a faster moving impurity), and then with PE/Et_2O 1/1. 17.38 g (57% yield) of pure compund Ii were recovered as an oil:EI- MS: m/z 201 (4, M-OtBu), 173 (2, M-Boc); Rf_B 0.44; RT_A 10.4; RT_B 19.1.

Step 10

Boc-Leu-H (Ij)

8.4 g (30.58 mmol) of Boc-Leu-N(CH_3)OCH_3 (Ii) were dissolved in 350 ml of anhydrous Et_2O and made to react at 0°C with 3.48 g (91.74 mmol) of $LiAlH_4$ added portionwise in 15 min. The reaction mixture was stirred for 15 min at 0°C, then 175 ml of AcOEt, followed by 700 ml of 10% citric acid, were added, keeping the reaction temperature at 0°C. After 30 min stirring the reaction mixture was extracted with AcOEt (5 x 300 ml), the combined organic layers were washed with 10% citric acid, then with brine to neutrality, and dried over Na_2SO_4. Evaporation of the solvent gave 6.26 g (95% yield) of crude oily compound Ij: EI-MS: m/z 186 (7, M-CHO); Rf_B 0.38; RT_A 7.7; RT_B 15.

Step 11

Boc-LeuΨ(CH_2NH)Met-NH_2 (Ik)

To a solution of 6.14 g (28.52 mmol) of Boc-Leu-H (Ij) in 100 ml of 1% AcOH in anhydrous MeOH, 4.24 g (28.52 mmol) of HCl · H-Met-NH_2 were added, followed by 4.21 g (57 mmol) of $NaBH_3CN$ added portionwise in 30 min at room temperature. After 40 min additional

stirring the solution was evaporated, the residue taken up in 300 ml of 5% $NaHCO_3$ and the product extracted with AcOEt (5 x 100 ml). The organic phase was washed with brine to neutrality, dried over Na_2SO_4 and concentrated. 5.30 g (53% yield) of pure compound Ik were obtained: m.p. 124-126°C; FD-MS: m/z 347 (100, $M^{+\cdot}$); Rf_B 0.16; RT_A 6.2; RT_B 12.4.

Step 12

H-LeuΨ(CH_2NH)Met-NH_2 . 2 HCl (Il)

A solution of 1.04 g (3 mmol) of Boc-LeuΨ(CH_2NH)Met-NH_2 (Ik) in 10 ml of 1.33 N HCl/AcOH, containing 1 ml of anisole and 0.5 ml of 2-mercaptoethanol, was stirred for 20 min at room temperature. Solvents were removed at reduced pressure and the oily residue was evaporated three times from DMF and once from MeOH, then it was triturated with AcOEt and Et_2O. 1.44 g (98.3% yield) of compound Il were obtained in two crops: EI-MS: m/z 247 (1, $M^{+\cdot}$), 203 (6, M-$CONH_2$) as free base; Rf_A 0.58; RT_B 3.6.

Step 13

Boc-His(Dnp)-LeuΨ(CH_2NH)Met-NH_2 (Im)

1.29 g (2.68 mmol) of Boc-His(Dnp)-OH·iPrOH were evaporated three times from DMF to remove the isopropyl alcohol of crystallization, then were dissolved in 15 ml of DMF, cooled at -25°C, and made to react with 0.30 ml (2.68 mmol) of NMM, followed by 0.27 ml (2.68 mmol) of ECC. After 2 min stirring at -12°C, a cold solution of 0.858 g (2.68 mmol) of H-LeuΨ(CH_2NH)Met-NH_2 · 2 HCl (Il) and 0.60 ml (5.36 mmol) of NMM in 15 ml of DMF were added. The reaction mixture was kept for 60 min at -12°C, then for 30 min at 0°C. The solvent was removed in vacuo and the residue was dissolved in AcOEt, washed with 5% $NaHCO_3$ and then brine to neutrality. After drying over Na_2SO_4, the solvent was evaporated and the oily residue purified by flash-chromatography on silica gel, eluting with AcOEt containing increasing amount of MeOH (from 0.5% to 10%). The product was recovered by evaporation of the solvents and trituration with Et_2O: 1.23 g (70.7% yield) of compound Im were obtained: m.p. 70°C (mod.) - 90°C (dec.); FD-MS: m/z 651 (100, MH^+); Rf_C 0.57; RT_A 12.0.

Step 14

H-His(Dnp)-LeuΨ(CH_2NH)Met-NH_2 . 2 HCl (In)

Starting from 1.16 g (1.78 mmol) of Boc-His(Dnp)-LeuΨ(CH_2NH)Met-NH_2 (Im), and operating as described in step 12, 1.09g (98% yield) of compound In were obtained from AcOEt: 110°C (mod.) - 200°C (dec.); FD-MS: m/z 551 (100, MH^+) as free base; Rf_A 0.41; RT_A 4.2; RT_B 7.1.

Step 15

Boc-Gln-Trp-Ala-Val-Gly-His(Dnp)-LeuΨ(CH_2NH)Met-NH_2 (Io)

156 mg (1.16 mmol) of HOBt, 239 mg (1.16 mmol) of DCC, 660 mg (1.06 mmol) of H-His(Dnp)-LeuΨ(CH_2NH)Met-NH_2 · 2 HCl (In) and 0.23 ml (2.12 mmol) of NMM were successively added to a solution of 700 mg (1.06 mmol) of Boc-Gln-Trp-Ala-Val-Gly-OH (Ih) in 8 ml of DMF. The reaction mixture was stirred at 0°C for 1 h and at room temperature overnight, then it was filtered and evaporated in vacuo. The oily residue was dissolved in DMF and poured with stirring into a 5% $NaHCO_3$ aqueous solution. The suspension was filtered and the product washed with water to neutrality. The crude material was purified by flash-chromatography in the eluent system composed by

AcOEt/MeOH 8/2. 820 mg (65% yield) of compound Io were obtained from MeOH/AcOEt/Et_2O: 128°C (mod.) - 145°C (dec.); FAB-MS: m/z 1192 (23, MH^+); Rf_C 0.10; RT_A 10.6.

Step 16

H-Gln-Trp-Ala-Val-Gly-His(Dnp)-Leuψ(CH_2NH)Met-NH_2 · 2 HCl (Ip)

800 mg (0.67 mmol) of Boc-Gln-Trp-Ala-Val-Gly-His(Dnp)-Leuψ(CH_2NH) Met-NH_2 (Io) were dissolved in a mixture of 8 ml of 1.33 N HCl/ AcOH, 0.8 ml of anisole and 0.4 ml of 2-mercaptoethanol, and made to react for 90 min at room temperature. The solvent were removed in vacuo and the residue was ground with Et_2O, giving 0.765 mg (98% yield) of compound Ip: m.p. 165°C (mod.) - 220°C (dec.); FAB-MS: m/z 1092 (6, MH^+) as free base; Rf_A 0.20; RT_A 4.5; RT_B 12.1.

Step 17

Boc-pMel-Gln-Trp-Ala-Val-Gly-His(Dnp)-LeuΨ(CH_2NH)Met-NH_2 (I)

321 mg (0.64 mmol) of Boc-pMel-OSu [prepared extemporaneously from 259 mg (0.64 mmol) of Boc-pMel-OH (see our UK Pat. Appl. N° 8906000.9), 77 mg (0.67 mmol) of HOSu and 132 mg (0.64 mmol) of DCC in 5 ml of DMF] were added dropwise to a cooled solution (0°C) of 500 mg (0.43 mmol) of H-Gln-Trp-Ala-Val-Gly-His(Dnp)-LeuΨ(CH_2NH) Met-NH_2 · 2 HCl (Ip) and 0.096 ml (0.87 mmol) of NMM in 10 ml of DMF. The reaction mixture was stirred overnight at room temperature, then it was poured dropwise into a 5% $NaHCO_3$ aqueous solution. The suspension was stirred for 10 min at room temperature, then filtered and washed with water to neutrality. The crude product (520 mg, 78% yield) was purified by preparative RP-HPLC, running a gradient from 80% to 100% of eluent B in eluent A over 20 min, with a flow rate of 30 ml/min. 286 mg (45% yield) of compound I were obtained: m.p. 140°C (mod.) - 170°C (dec.); AA ratios: Glu 1, Gly 0.93 (1), Ala 0.98 (1), Val 1.00 (1) (pMel, Trp, His(Dnp) and LeuΨ(CH_2NH)Met-NH_2 n.d.); FAB-MS: m/z 1478 (10, MH^+); Rf_A 0.50; RT_A 27.0.

Example 2

Preparation of

Boc-pMel-Gln-Trp-Ala-Val-Gly-His-LeuΨ(CH_2NH)Met-NH_2 (II)

180 mg (0.12 mmol) of Boc-pMel-Gln-Trp-Ala-Val-Gly-His(Dnp)-Leu Ψ(CH_2NH)Met-NH_2 (I) were suspended in 7.2 ml of 0.02 M KH_2PO_4 (brought to pH 8 with 1N NaOH), then 7.2 ml of 2-mercaptoethanol were added. The resulting solution was stirred for [illegible] min at room temperature, then it was concentrated in vacuo and poured dropwise into Et_2O. The crude product was filtered and purified first by flash-chromatography on silica gel, in the solvent system AcOEt/ MeOH 7/3 v/v; then by preparative RP-HPLC, running a gradient from 30% to 90% of elunt B in eluent A over 20 min, with a flow rate of 24 ml/min. 82 mg (52% yield) of compound II were obtained: m.p. 75°C (mod.) - 120° (dec.); AA ratios: Glu 1, Gly 0.97 (1), Ala 0.99 (1), Val 1.02 (1), His 0.94 (1) (pMel, Trp and LeuΨ(CH_2NH)Met-NH_2 n.d.); FAB-MS: m/z 1312 (7, MH^+); Rf_A 0.14: RT_A 18.1.

Example 3

Preparation of

Ac-pMel-Gln-Trp-Ala-Val-Gly-His(Dnp)-Leuψ(CH$_2$NH)Met-NH$_2$ (III)

Step 1

Ac-pMel-OH (IIIa)

A solution of 0.991 mg (9 mmol) of acetyl imidazole in 10 ml of DMF was dropped with stirring into a solution of 500 mg (1.5 mmol) of H-pMel-OH (SIGMA) in 10 ml of DMF. The reaction mixture was stirred for 5 h at room temperature, then the solvent was evaporated in vacuo. The crude material was purified through its DCHA salt. 312 mg (60% yield) of compound III a were obtained from AcOEt/Et$_2$O: m.p. 52-54°C; EI-MS: m/z 346 (2, $m^{+\cdot}$); Rf_D 0.33; RT_B 12.8.

Step 2

Ac-pMel-Gln-Trp-Ala-Val-Gly-His(Dnp)-Leuψ(CH$_2$NH)Met-NH$_2$ (III)

70 mg (0.2 mmol) of Ac-pMel-OH were dissolved in 5 ml of DMF, then 233 mg (0.2 mmol) of H-Gln-Trp-Ala-Val-Gly-His(Dnp)-Leu (CH$_2$NH) Met-NH$_2$ · 2 HCl (Ip) were added, followed, at 5°C, by 0.066 ml (0.6 mmol) of NMM and 88.5 mg (0.2 mmol) of BOP. The reaction mixture was stirred at room temperature for 4.5 h, then it was poured

dropwise into AcOEt. The crude product was filtered, washed with AcOEt and purified by preparative RP-HPLC, running a gradient from 60% to 90% of eluent B in eluent A over 40 min, with a flow rate of 24 ml/min. 128 mg of compound III (45% yield) were obtained: m.p. 124-150°C (dec.); AA ratios: Glu 1, Gly 0.89 (1), Ala 0.98 (1), Val 0.94 (1), (Trp, His(Dnp) and LeuΨ(CH_2NH)Met-NH_2 n.d.); FAB-MS: m/z 1420 (16, MH^+); Rf_A 0.57; RT_A 18.15.

Example 4

Preparation of

Cab-Gln-Trp-Ala-Val-Gly-His(Dnp)-LeuΨ(CH_2NH)Met-NH_2 (IV)

Starting from 0.20 g (0.172 mmol) of H-Gln-Trp-Ala-Val-Gly-His(Dnp) LeuΨ(CH_2NH)Met-NH_2 · 2 HCl (Ip), 0.068 g (0.258 mmol) of [p-bis(2-chloroethyl)amino]benzoic acid (Cab-OH), 0.115 g (0.258 mmol) of BOP and 0.057 ml (0.516 mmol) of NMM, and operating as described for the preparation of compound III, a crude material was obtained, which was purified by preparative RP-HPLC, running a gradient from 30% to 90% of eluent B in eluent A over 20 min, with a flow rate of 24 ml/min. 0.138 g (60% yield) of compound III were obtained: m.p. 128-150°C (dec.); AA ratios: Glu 1.02 (1), Gly 1, Ala 1.00 (1), Val 0.95 (1) (Trp, His(Dnp) and LeuΨ(CH_2NH)Met-NH_2 n.d.); FAB- MS: m/z 1336 (13, MH^+); Rf_A 0.47; RT_A 19.9.

Example 5

Preparation of

Cab-Gln-Trp-Ala-Val-Gly-His-Leuψ(CH_2NH)Met-NH_2 (V)

0.20 g (0.15 mmol) of Cab-Gln-Trp-Ala-Val-Gly-His(Dnp)-Leuψ(CH_2NH) Met-NH_2 (IV) were suspended in 10.5 ml of 0.1 M KH_2PO_4 (brought to pH 8.1 with 1N KOH), then 10.5 ml of 2-mercaptoethanol were added. The resulting solution was stirred for 30 min at room temperature, then it was concentrated in vacuo. The product was extracted with BuOH, and the organic layer was washed twice with water and evaporated. The residue was dissolved in MeOH and precipitated with Et_2O. The crude product was purified by preparative RP-HPLC, running a gradient from 50% to 90% of eluent B in eluent A over 30 min, with a flow rate of 24 ml/min: 96 mg (55% yield) of compound V were obtained: m.p. 128-150°C (dec.); AA ratios: Glu 1.08 (1), Gly 1, Ala 0.90 (1), Val 0.91 (1), Trp 1.10 (1), His 1.09 (1) (Leu (CH_2NH) Met NH_2 n.d.); FAB-MS: m/z 1170 (23, MH^+); Rf_A 0.39; RT_A 14.1.

Operating as described in the previous examples, the following peptides were also prepared:

H-pMel-Gln-Trp-Ala-Val-Gly-Leuψ(CH_2NH)Leu-NH_2

H-pMel-Gln-Trp-Ala-Val-Gly-Leuψ(CH_2NH)Nle-NH_2

Ac-pMel-Gln-Trp-Ala-Val-Gly-Leuψ(CH_2NH)Nle-NH_2

Ac-pMel-phe-Gln-Trp-Ala-Val-Gly-Leuψ(CH_2NH)Nle-NH_2

Boc-pMel-phe-Gln-Trp-Ala-Val-Gly-Leuψ(CH_2NH)Nle-NH_2

Boc-pMel-Gln-Trp-Ala-Val-Gly-Leu ψ (CH_2NH)Nle-NH_2

H-pMel-phe-Gln-Trp-Ala-Val-Gly-Leuψ(CH_2NH)Nle-NH_2

Cab-Gln-Trp-Ala-Val-Gly-Leu ψ (CH_2NH)Met-NH_2

Cab-Gln-Trp-Ala-Val-Gly-Leu ψ (CH_2NH)Leu-NH_2

Cab-Gln-Trp-Ala-Val-Gly-Leu ψ (CH_2NH)Nle-NH_2

Cab-phe-Gln-Trp-Ala-Val-Gly-Leu ψ (CH_2NH)Met-NH_2

Cab-phe-Gln-Trp-Ala-Val-Gly-Leu ψ (CH_2NH)Leu-NH_2

Cab-phe-Gln-Trp-Ala-Val-Gly-Leu ψ (CH_2NH)Nle-NH_2

CLAIMS

1. A peptide of the formula I:

R—A—B—C—Trp—Ala—Val—X—Y—T—W I

wherein

R represents a group of the formula

$4\text{-}(ClCH_2CH_2)_2N\text{-}C_6H_4\text{-}CH_2CH(NHR_1)CO\text{-}$;

$3\text{-}(ClCH_2CH_2)_2N\text{-}C_6H_4\text{-}CH_2CH(NHR_1)CO\text{-}$;

$4\text{-}(ClCH_2CH_2)_2N\text{-}C_6H_4\text{-}CO\text{-}$; $3\text{-}(ClCH_2CH_2)_2N\text{-}C_6H_4\text{-}CO\text{-}$;

$ClCH_2CH_2NHCO\text{-}$; $ClCH{=}CH\text{-}CO\text{-}$, $BrCH{=}CH\text{-}CO\text{-}$, $CH_2{=}CClCO\text{-}$,

$CH_2{=}CBrCO\text{-}$ (either <u>cis</u> or <u>trans</u> isomers);

$CH_2\text{-}CH\text{-}CH_2\text{-}CO\text{-}$; $CH \equiv C\text{-}CO\text{-}$; $ClCH_2CH_2CH_2N(NO)CO\text{-}$;
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$ClCH_2CO\text{-}CH(R_2)NHCO(CH_2)_2CO\text{-}$;

A represents a valence bond, or a Gly, Leu-Gly, Arg-Leu-Gly, or Gln-Arg-Leu-Gly residue,

B represents a valence bond or a Asn, phe or Thr residue;

C represents a Gln or His residue,

X represents a Gly or ala residue;

Y represents a valence bond, or a His(R_3), his(R_3), Phe, phe, Ser, ser, Ala or ala residue;

T represents a valence bond, or a Leu, leu, Phe or phe residue;

W represents a group of the formula OR_2, NH_2, $NH(CH_2)_4CH_3$, $NH(CH_2)_2C_6H_5$, Met-R_4, Leu-R_4, Ile-R_4, or Nle-R_4;

R_1 represents a hydrogen atom, a Boc group or a C_1-C_{11} acyl group,

R_2 represents a hydrogen atom, a linear or branched alyphatic chain having from 1 to 11 carbon atoms, a benzyl or a phenyl group,

R_3 represents a hydrogen atom or a Tos, Dnp or Bzl group,

R_4 represents NH_2, $NH-NH_2$ or OR_2,

one or more peptide bonds (CONH) are replaced by reduced peptide bonds (CH_2NH), and the pharmaceutically acceptable salts thereof.

2. A peptide of the formula I according to claim 1 wherein R represents pMel or Cab, R_1 represents hydrogen atom, Boc or acetyl group, A and Y represent valence bonds, B represents a valence bond or a phe residue, C represents a Gln residue, X represents a His (Dnp), His or Gly residue, T represents a Leu residue, W represents a group of the formula Leu-NH_2 or Nle-NH_2 and the reduced peptide bond (CH_2NH) is that between T and W.

3. A pharmaceutical composition comprising a peptide according to claim 1 or 2 or a pharmaceutically acceptable salt of such a peptide in admixture with a pharmaceutically acceptable diluent or carrier.

4. A process for the preparation of a peptide according to claim 1 or 2, the process comprising condensing amino acids and/or amino acid derivatives in the desired sequence and/or peptide fragments containing these amino acids or their derivatives in the desired sequence to give the desired peptide, the end carboxylic acid group being activated for the peptide linkage and the remaining groups being protected and deprotecting the resultant compound and/or converting the resultant peptide into a pharmaceutically acceptable salt thereof.

5. The use of peptides according to claims 1 and 2 for the preparation of a pharmaceutical suitable for therapy of human neoplasms.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 90/01836

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indic- te all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 7/02, A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	EP, A2, 0309297 (THE ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND) 29 March 1989, see the whole document --	1-5
P,Y	WO, A1, 9001037 (FARMITALIA CARLO ERBA S.R.L.) 8 February 1990, see the whole document --	1-5
P,Y	WO, A1, 9003980 (ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND) 19 April 1990, see the whole document --	1-5

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25th January 1991	18. 02. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	miss T. MORTENSEN

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A1, 0339193 (FARMITALIA CARLO ERBA S.R.L.) 2 November 1989, see the whole document	1-5

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/EP 90/01836

SA 41096

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 28/12/90
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0309297	29/03/89	AU-D- 2710288	18/04/89
		JP-T- 2502016	05/07/90
		WO-A- 89/02897	06/04/89
WO-A1- 9001037	08/02/90	AU-D- 3878889	19/02/90
		EP-A- 0380623	08/08/90
WO-A1- 9003980	19/04/90	AU-D- 4494989	01/05/90
		AU-D- 3414689	16/10/89
		WO-A- 89/09231	05/10/89
EP-A1- 0339193	02/11/89	JP-A- 2015096	18/01/90

For more details about this annex : see Official Journal of the European patent Office, No. 12/82